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Effects of DL-2-bromopalmitoyl-CoA and bromoacetyl-CoA in rat liver and heart mitochondria

Inhibition of carnitine palmitoyltransferase and displacement of [^{14}C]malonyl-CoA from mitochondrial binding sites

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1. The overt form of carnitine palmitoyltransferase (CPT_1) in rat liver and heart mitochondria was inhibited by DL-2-bromopalmitoyl-CoA and bromoacetyl-CoA. *S*-Methanesulphonyl-CoA inhibited liver CPT_1. 2. The inhibitory potency of DL-2-bromopalmitoyl-CoA was 17 times greater with liver than with heart CPT_1. Inhibition of CPT_1 by DL-2-bromopalmitoyl-CoA was unaffected by 5,5′-dithiobis-(2-nitrobenzoic acid) or (in liver) by starvation. 3. In experiments in which DL-2-bromopalmitoyl-CoA displaced [^{14}C]malonyl-CoA bound to liver mitochondria, the K_D (competing) was 25 times the IC_{50} for inhibition of CPT_1 providing evidence that the malonyl-CoA-binding site is unlikely to be the same as the acyl-CoA substrate site. 4. Bromoacetyl-CoA inhibition of CPT_1 was more potent in heart than in liver mitochondria and was diminished by 5,5′-dithiobis-(2-nitrobenzoic acid) or (in liver) by starvation. Bromoacetyl-CoA displaced bound [^{14}C]malonyl-CoA from heart and liver mitochondria. In heart mitochondria this displacement was competitive with malonyl-CoA and was considerably facilitated by L-carnitine. In liver mitochondria this synergism between carnitine and bromoacetyl-CoA was not observed. It is suggested that bromoacetyl-CoA interacts with the malonyl-CoA-binding site of CPT_1. 5. L-Carnitine also facilitated the displacement by DL-2-bromopalmitoyl-CoA of [^{14}C]malonyl-CoA from heart, but not from liver, mitochondria. 6. DL-2-Bromopalmitoyl-CoA and bromoacetyl-CoA also inhibited overt carnitine octanoyltransferase in liver and heart mitochondria. 7. These findings are discussed in relation to inter-tissue differences in (*a*) the response of CPT_1 activity to various inhibitors and (*b*) the relationship between high-affinity malonyl-CoA-binding sites and those sites for binding of L-carnitine and acyl-CoA substrates.

CPT_1 in liver (McGarry *et al.*, 1978) and several other mammalian tissues (Saggerson & Carpenter, 1981*b*, 1982*a*; Bird *et al.*, 1985) is potently inhibited by malonyl-CoA. Other short-chain thioesters also inhibit the enzyme, with different potencies (Mills *et al.*, 1983, 1984). Malonyl-CoA binds to high-affinity binding sites on mitochondria from skeletal muscle, liver, heart and brain (Mills *et al.*, 1983, 1984; Bird & Saggerson, 1984; Zammit *et al.*, 1984; Bird *et al.*, 1985), and it is thought that occupancy of these binding sites leads to inhibition of CPT_1. Although there is no conclusive proof at present, several lines of evidence have been advanced suggesting that these high-affinity sites may represent a regulatory locus that is distinct from the acyl-CoA substrate-binding site of the enzyme (Mills *et al.*, 1984; Bird & Saggerson, 1984).

Several studies show that CPT_1 in liver differs

Abbreviations used: COT, carnitine acyltransferase activity with octanoyl-CoA; CPT_1, the overt form of carnitine palmitoyltransferase (EC 2.3.1.21); I_{50}, the concentration of displacing ligand causing 50% of maximal displacement of [^{14}C]malonyl-CoA; $I_{max.}$, the percentage inhibition of CPT_1 or overt COT at infinite malonyl-CoA concn.; IC_{50}, the concentration of malonyl-CoA causing 50% of $I_{max.}$; Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid).

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from the heart enzyme. Differences include: the effects of $[K^+]$ and $[Mg^{2+}]$ (Saggerson, 1982), sensitivity to malonyl-CoA (Saggerson & Carpenter, 1981*b*; Mills *et al.*, 1983, 1984; Cook, 1984), K_D values for high-affinity binding of malonyl-CoA to mitochondria (Bird & Saggerson, 1984), the apparent K_m for carnitine (McGarry *et al.*, 1983; Mills *et al.*, 1984) and long-term adaptive changes in both specific activity and sensitivity to malonyl-CoA, which are seen in liver but not in heart (Saggerson & Carpenter, 1981*a*; Veerkamp & Van Moerkerk, 1982). It is not known at present whether these differences reflect the presence of tissue-specific CPT_1 isoenzymes.

Mitochondria from mammalian tissues also contain an overt medium-chain carnitine acyltransferase activity (Markwell *et al.*, 1977; Choi *et al.*, 1977; Saggerson & Carpenter, 1981*b*). The acyl-chain-length specificity of this proposed enzyme is likely to overlap with those of carnitine acetyltransferase and CPT. Clarke & Bieber (1981) have proposed that heart COT is attributable to these latter activities, and is unlikely to be a distinct enzyme. It is still unresolved whether liver mitochondria contain a distinct overt COT enzyme, although it has been demonstrated that liver overt COT and CPT_1 activities differ in several respects, e.g. response to $[K^+]$ and $[Mg^{2+}]$, the effect of Nbs_2, sensitivity to malonyl-CoA and kinetics with respect to [acyl-CoA] (Saggerson & Carpenter, 1981*a*,*c*, 1982*b*; Saggerson, 1982).

In the present study we have investigated the effects of bromoacetyl-CoA and 2-bromopalmitoyl-CoA on CPT_1 and overt COT activities and on [^{14}C]malonyl-CoA binding in heart and liver mitochondria. The findings provide further information both about inter-tissue differences in CPT_1 activities and about the relationship between the malonyl-CoA-binding site and the catalytic site of CPT_1.

Materials and methods

Animals and chemicals

Sources of these were as described by Saggerson (1982) and Bird & Saggerson (1984). In addition, bromoacetyl chloride, methanesulphonyl chloride, 2-bromopalmitic acid, oxalyl chloride and *NN*-dimethylformamide were from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Chemical synthesis

2-Bromopalmitoyl-CoA, bromoacetyl-CoA and methanesulphonyl-CoA were prepared from acid chlorides and CoA (Seubert, 1960). 2-Bromopalmitoyl-CoA was synthesized from 2-bromopalmitic acid via 2-bromopalmitoyl chloride as described by Chase & Tubbs (1972). For methanesulphonyl-CoA, the method of Owens *et al.* (1981) was modified in that 10mg of CoA were made to react at 0°C with 10μl of methanesulphonyl chloride in 1M-$KHCO_3$, pH8.0, with vigorous shaking for 30s. The product, after diethyl ether extractions, was not further purified. Bromoacetyl-CoA was prepared by a similar method, but with 10mg of CoA and 10μl of bromoacetyl chloride. All three acyl-CoAs were stored at −20°C as aqueous solutions, pH3–5. Concentrations were determined from u.v. spectra, assuming $\varepsilon_{260} = 14.6 \times 10^3$. In addition, 2-bromopalmitoyl-CoA was assayed enzymically by measuring the release of CoA catalysed by a partially purified rat brain palmitoyl-CoA hydrolase (EC 3.1.2.2) (Anderson & Erwin, 1971) in a Nbs_2-linked assay (Knauer, 1979). This method gave similar values to those obtained from A_{260} measurements. The contamination with free thiol was determined by reaction with Nbs_2 at pH8, and was less than 1% for methanesulphonyl-CoA and 2-bromopalmitoyl-CoA, and approx. 5% for bromoacetyl-CoA.

Isolation of mitochondria

Rat liver and heart mitochondria were obtained as described by Saggerson (1982) and finally suspended in 0.3M-sucrose medium containing 10mM-Tris/HCl buffer (pH7.4) and 1mM-EGTA. Mitochondrial protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Enzyme assays

CPT_1 and overt COT activities were assayed at 25°C in intact freshly prepared mitochondria by a radiochemical method (Saggerson, 1982). The assays contained 25mM-Tris/HCl buffer (pH7.4), 150mM-sucrose, 60mM-KCl, fatty acid-poor albumin (1.3mg/ml), 1mM-EGTA and (unless otherwise stated) 40μM-palmitoyl-CoA or 40μM-octanoyl-CoA. Mitochondria were preincubated in this medium for 2min before addition of 400μM-L-carnitine and DL-[*methyl*-^{3}H]carnitine (1μCi/ml), giving a final volume of 1ml. After 4min the reaction was terminated, and incorporation of ^{3}H into butan-1-ol-soluble products determined as described by Saggerson *et al.* (1982). The amounts of mitochondrial protein per assay were: liver (fed state), 0.18–0.28mg; liver (starved state), 0.16–0.21mg; heart, 0.1–0.25mg. Additions of malonyl-CoA, 2-bromopalmitoyl-CoA, bromoacetyl-CoA, *S*-methanesulphonyl-CoA and Nbs_2 were made immediately before addition of the mitochondria. Dithiothreitol was omitted from these assays because of its reactivity with the last four compounds.

Binding of [^{14}C]malonyl-CoA to mitochondria

Specific binding of [2-^{14}C]malonyl-CoA to mitochondria was measured as described by Bird & Saggerson (1984). Liver mitochondria or heart mitochondria were incubated with [2-^{14}C]malonyl-CoA at 0–4°C for 20min in 1.0ml containing 25mM-Tris/HCl buffer (pH7.4), 150mM-sucrose, 60mM-KCl, fatty acid-poor albumin (1.3mg/ml), 1mM-EGTA and other additions as indicated. Again, dithiothreitol was omitted from these assays, and additions of 2-bromopalmitoyl-CoA or bromoacetyl-CoA were made immediately before addition of mitochondria. Non-specific entrapment of [^{14}C]malonyl-CoA was taken as the radioactivity remaining bound in the presence of 0.5mM unlabelled malonyl-CoA and was subtracted from all values to give the amount of specifically bound malonyl-CoA.

Analysis of results

The maximum inhibitions ($I_{max.}$) of CPT_1 and overt COT activities were determined from graphs of (% inhibition)$^{-1}$ versus [inhibitor]$^{-1}$ by extrapolation to infinite [inhibitor] (Saggerson & Carpenter, 1981*a*). Values for IC_{50} were also determined from these graphs, by using linear regression, where they were linear. In other cases IC_{50} values were read from the graphs of % inhibition versus [inhibitor]$^{-1}$ after subtraction of non-inhibitable activity. Statistical significance was determined by using Student's *t* test for unpaired samples.

Results and discussion

Inhibition of CPT_1 by DL-*2-bromopalmitoyl-CoA, bromoacetyl-CoA and S-methanesulphonyl-CoA*

CPT activity was measured in the direction of palmitoylcarnitine formation in an iso-osmotic medium, by using intact mitochondria. Under these conditions the measured CPT activity corresponded to the overt pool, or CPT_1. This was verified by observing the effect of malonyl-CoA, which commonly gave approx. 90% of maximal inhibition in both heart and liver mitochondria, with IC_{50} values similar to those previously reported under similar assay conditions (Saggerson & Carpenter, 1981*a*,*b*). DL-2-Bromopalmitoyl-CoA is an established inhibitor of palmitoyl-CoA oxidation by rat liver mitochondria (Chase & Tubbs, 1972) and of soluble CPT_1 from rat liver (Hoppel & Tomec, 1972) and ox liver (West *et al.*, 1971). It is presumed that this inhibition is at the catalytic site, and it is thought (West *et al.*, 1971; Chase & Tubbs, 1972) that the mechanism of inhibition is similar to the carnitine-dependent inhibition of carnitine acetyltransferase by bromoacetyl-CoA (Chase & Tubbs, 1969). Potent inhibition of CPT_1 in liver mitochondria by 2-bromopalmitoyl-CoA (Fig. 1*a*) was therefore expected. Previous work has indicated that this inhibition would not be readily reversible on dilution (West *et al.*, 1971). We have confirmed this in an experiment in which liver mitochondria from a fed rat were preincubated at 0–4°C with 400μM-carnitine and 0, 2μM- or 10μM-DL-2-bromopalmitoyl-CoA. After 2 min these incubations were diluted 20-fold and CPT_1 assays performed with 40μM-palmitoyl-CoA. The resulting inhibitions were 45% and 88% after preincubation with 2μM- and 10μM-2-bromopalmitoyl-CoA respectively. These values may be contrasted with 19% and 54% inhibition obtained when the liver enzyme was assayed directly with 0.1μM and 0.5μM-2-bromopalmitoyl-CoA respectively without preincubation. The IC_{50} value for the liver enzyme was unaltered on starvation (Table 1) and was similar to that for inhibition of palmitoyl-CoA oxidation by rat liver mitochondria (Tubbs *et al.*, 1980). The absolute numerical values should, however, be treated with some caution, since the inhibitor presumably is largely bound to the albumin in the assay. It was most surprising to find that CPT_1 in intact heart mitochondria was inhibited far less potently by 2-bromopalmitoyl-CoA (Fig. 1*a*), with an IC_{50} value approx. 17 times higher than for liver (Table 1). This difference could not be accounted for by changes in assay conditions, since the CPT_1 activities and mitochondrial protein concentrations in the assays were similar for heart mitochondria and for those from liver of starved rats. Although other explanations are possible, this finding is consistent with heart and liver containing different CPT_1 isoenzymes. 2-Bromopalmitate inhibits palmitate oxidation in intact heart and in hepatocytes (Burgess *et al.*, 1968; Sauer *et al.*, 1971), but there is insufficient information to indicate whether there are inter-tissue differences in the effects of the inhibitor at this level.

Bromoacetyl-CoA, in the presence of L-carnitine, is an active-site-directed inhibitor of carnitine acetyltransferase (Chase & Tubbs, 1969). Bromoacetyl-CoA has not previously been investigated as an inhibitor of CPT, except by Miyazawa *et al.* (1983), who reported that, like malonyl-CoA, it was ineffective against solubilized purified rat liver CPT. Since CPT_1 in mitochondria from rat heart or liver can, in addition to malonyl-CoA, be inhibited by other short-chain acyl-CoA esters (succinyl-CoA, methylmalonyl-CoA, propionyl-CoA), it was decided to test the effect of bromoacetyl-CoA against the enzyme *in situ* in intact mitochondria. It was also considered poss-

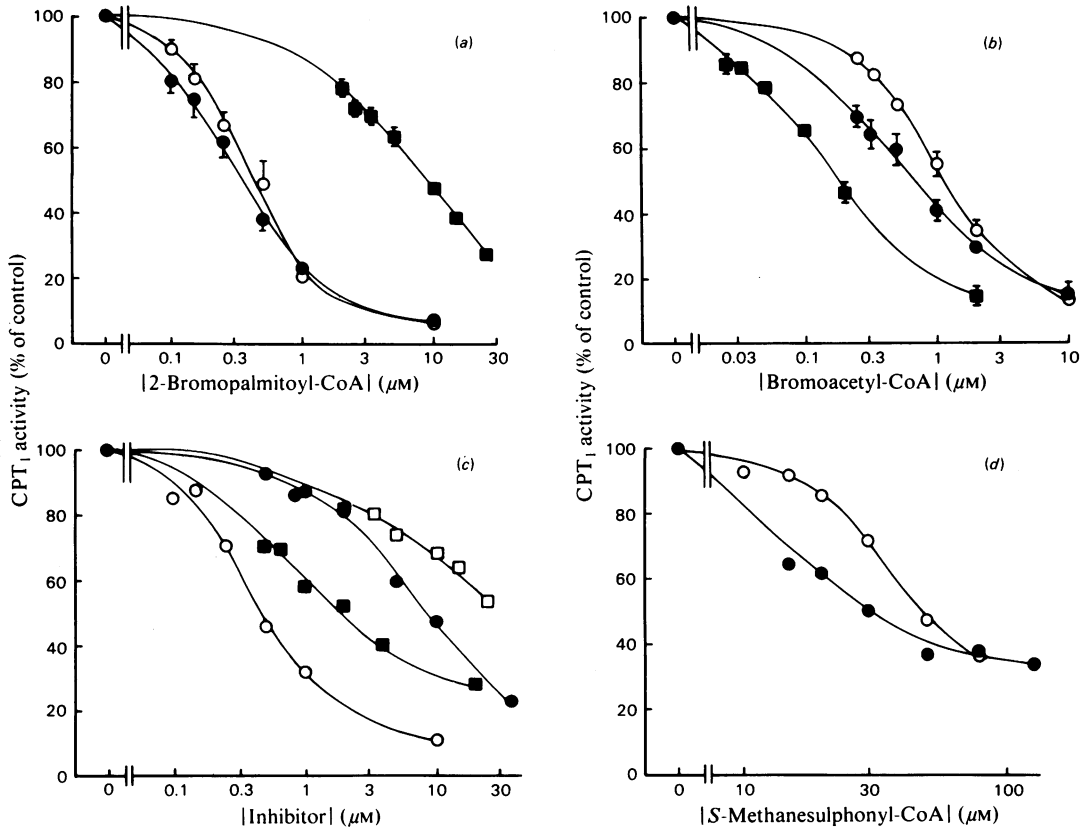

Fig. 1. *Effects of* DL-*2-bromopalmitoyl-CoA, bromoacetyl-CoA and S-methanesulphonyl-CoA on* CPT_1 *activities*
CPT_1 assays were performed as described in the Materials and methods section. L-Carnitine and palmitoyl-CoA were present at 400 μM and 40 μM respectively. CPT_1 activities with these substrates alone were 4.83 ± 0.45, 9.47 ± 0.53 and 9.80 ± 1.44 nmol/min per mg of protein for mitochondria from fed rat liver, 24h-starved-rat liver and heart respectively. Nbs_2 (150 μM) increased this rate by 7% in liver mitochondria and decreased this rate by 40% in heart mitochondria. All values are expressed as percentages of these activities in the absence of inhibitory acyl-CoAs. In (*a*) and (*b*) the bars indicate S.E.M.; where not shown these lie within the symbol. The values in brackets indicate the numbers of separate experiments. (*a*) ●, Liver (fed) [4]; ○, liver (24h starved) [3]; ■, heart (fed) [5]. (*b*) Symbols as for (*a*) [3 in each case]. (*c*) 150 μM-Nbs_2 was present throughout; all mitochondria were from fed animals: ○, liver with 2-bromopalmitoyl-CoA [2]; ●, liver with bromoacetyl-CoA [2]; □, heart with 2-bromopalmitoyl-CoA [2]; ■, heart with bromoacetyl-CoA [2], (*d*) ●, Liver (fed) [2]; ○, liver (starved) [2].

Table 1. IC_{50} *and* $I_{max.}$ *values for inhibition of* CPT_1 *by* DL-*2-bromopalmitoyl-CoA, bromoacetyl-CoA and S-methanesulphonyl-CoA*

The values for each experiment were calculated from the data of Figs. 1(*a*)–(*d*) as described in the Materials and methods section, and are means ± S.E.M. where there were more than two determinations. The numbers of experiments are shown in brackets. * indicates significantly different IC_{50} value ($P < 0.01$) from that found in fed liver.

Inhibitors and other additions	Liver (fed)		Liver (24h starved)		Heart (fed)	
	IC_{50}(μM)	$I_{max.}$ (%)	IC_{50}(μM)	$I_{max.}$ (%)	IC_{50}(μM)	$I_{max.}$ (%)
DL-2-Bromopalmitoyl-CoA	0.36 ± 0.06	97 ± 3 [4]	0.43 ± 0.08	96 ± 1 [3]	6.8 ± 1.0*	89 ± 3 [5]
DL-2-Bromopalmitoyl-CoA + Nbs_2 (150 μM)	0.40	91 [2]	—	—	5.3	51 [2]
Bromoacetyl-CoA	0.49 ± 0.07	88 ± 3 [3]	1.14 ± 0.12*	95 ± 2 [3]	0.14 ± 0.01*	87 ± 6 [3]
Bromoacetyl-CoA + Nbs_2 (150 μM)	5.3	86 [2]	—	—	0.93	73 [2]
S-Methanesulphonyl-CoA	18	77 [2]	37	74 [2]	—	—

ible that specific interaction of this compound with the malonyl-CoA binding site could lead to an irreversible binding, which would be useful in future identification of the site. However, no evidence could be obtained for irreversible, time-dependent, inhibition of CPT from liver (results not shown) or heart (for example, see Table 3) in experiments where mitochondria were preincubated with the inhibitor before assay. As shown in Fig. 1(*b*), bromoacetyl-CoA was a very effective inhibitor of both heart and liver CPT_1 with IC_{50} values in the range 0.1–1 μM (Table 1), these being one to two orders of magnitude lower than the values for inhibition by acetyl-CoA (Mills *et al.*, 1983). Unlike DL-2-bromopalmitoyl-CoA, but like malonyl-CoA (Saggerson & Carpenter, 1981*a*,*b*), the IC_{50} of bromoacetyl-CoA for CPT_1 was significantly increased in liver on starvation and was lower in heart than in liver. The effect of bromoacetyl-CoA showed several other similarities to the effect of malonyl-CoA. First, the effect of bromoacetyl-CoA was abolished when CPT was solubilized with Triton X-100 (results not shown). The effect of malonyl-CoA is also lost when heart or liver CPT is solubilized (McGarry *et al.*, 1978; Saggerson, 1982). Second, as seen with malonyl-CoA (Saggerson & Carpenter, 1982*b*), the sensitivity of liver or heart CPT_1 to bromoacetyl-CoA was considerably decreased by Nbs_2 (Fig. 1*c*; Table 1). Nbs_2, however, had no effect on the potency of inhibition of the liver or heart enzyme by 2-bromopalmitoyl-CoA. Third, bromoacetyl-CoA increased the $s_{0.5}$ for palmitoyl-CoA without changing the maximum velocity and increased the sigmoidicity of the kinetics with respect to [palmitoyl-CoA] (Fig. 2). Malonyl-CoA has a similar effect on CPT_1 in liver (Saggerson & Carpenter, 1981*c*; Cook, 1984) and heart (Saggerson, 1982) mitochondria. It is therefore suggested that bromoacetyl-CoA interacts with the same site as malonyl-CoA. To test this hypothesis further, the inhibition by mixtures of bromoacetyl-CoA and malonyl-CoA was investigated in experiments where the proportions of the two inhibitors were held constant while their concentrations were varied over a wide range. As shown in Fig. 3, the plots of (percentage inhibition)$^{-1}$ versus [inhibi-

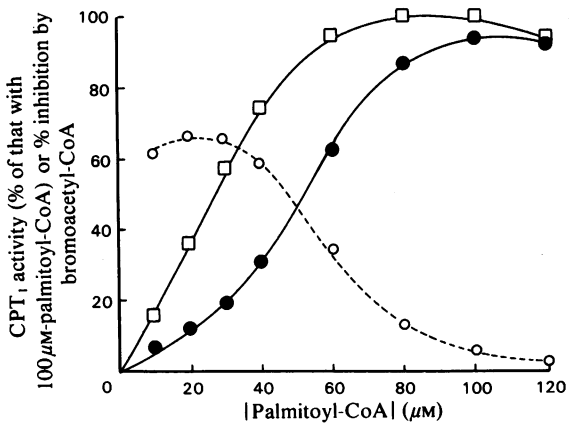

Fig. 2. *Inhibition of heart CPT_1 by bromoacetyl-CoA at different [palmitoyl-CoA]*

CPT_1 assays were performed with heart mitochondria as described in the Materials and methods section with 400 μM-L-carnitine and the indicated [palmitoyl-CoA]. The values are the means of two experiments and are expressed as percentages of the control activity with 100 μM-palmitoyl-CoA. □, CPT_1 activity without inhibitor; ●, CPT_1 activity with 0.25 μM-bromoacetyl-CoA; ○, percentage inhibition by bromoacetyl-CoA.

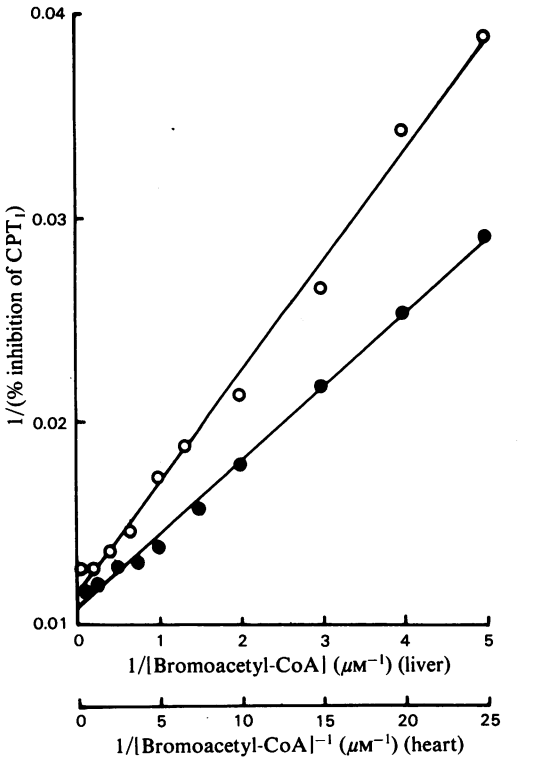

Fig. 3. *Inhibition of CPT_1 in liver and heart mitochondria from fed rats by mixtures of malonyl-CoA and bromoacetyl-CoA*

CPT_1 assays were performed as described in the Materials and methods section. L-Carnitine and palmitoyl-CoA were present at 400 μM and 40 μM respectively. The ratios [malonyl-CoA]/[bromoacetyl-CoA] were constant for each type of mitochondria and were 10:1 for liver (●) and 1:1 for heart (○). The values are from a single experiment.

tors]$^{-1}$ were linear for both liver and heart CPT_1, as predicted for two inhibitors competing for the same inhibitory site (Yonetani, 1982). Similar experiments were not attempted for mixtures of bromopalmitoyl-CoA and malonyl-CoA or bromoacetyl-CoA, because inhibition by bromopalmitoyl-CoA was not reversible and because [free bromopalmitoyl-CoA] may not increase linearly with [total bromopalmitoyl-CoA] in the presence of albumin.

S-Methanesulphonyl-CoA is an active-site-directed inhibitor of succinic thiokinase and 3-hydroxyacyl-CoA dehydrogenase (Owens *et al.*, 1981). It has been suggested that, because of the high specificity of the thiolsulphonate group for thiols, *S*-methanesulphonyl-CoA could be used to detect the presence of a reactive thiol in an acyl-CoA-binding site (Owens *et al.*, 1981). *S*-Methanesulphonyl-CoA was found to inhibit liver CPT_1 (Fig. 1*d*) and, like bromoacetyl-CoA and malonyl-CoA, appeared to show an increased IC_{50} on starvation. The IC_{50} values (Table 1) were similar to those for CoA (Mills *et al.*, 1983) and comparable with the inhibition of succinic thiokinase by *S*-methanesulphonyl-CoA (Owens *et al.*, 1981). In addition, 100μM-Nbs_2 abolished the inhibitory effect of 50μM-*S*-methanesulphonyl-CoA (results not shown). Although *S*-methanesulphonyl-CoA appeared to act in a similar manner to malonyl-CoA or bromoacetyl-CoA, the $I_{max.}$ values were less and the IC_{50} values much higher with this compound than with bromoacetyl-CoA, and no evidence could be obtained for irreversible time-dependent inhibition; therefore investigations with *S*-methanesulphonyl-CoA were not pursued further.

Inhibition of overt COT by DL-*2-bromopalmitoyl-CoA and bromoacetyl-CoA*

The inhibition of overt COT activity in liver and heart mitochondria by 2-bromopalmitoyl-CoA (Fig. 4*a*) was very similar to the pattern observed for CPT_1 (Fig. 1*a*). The IC_{50} values (Table 2) were not significantly different from the corresponding values for CPT_1 (Table 1), and there was no significant change in the sensitivity to this inhibitor of liver COT on starvation. Again, as with CPT_1, the heart enzyme showed a surprising insensitivity to the effect of 2-bromopalmitoyl-CoA. The similar IC_{50} values for overt COT and CPT_1 may be coincidental, since octanoyl-CoA probably binds much less tightly than palmitoyl-CoA to the albumin in the assays. The maximum inhibitable overt COT activities, with 2-bromopalmitoyl-CoA, were always relatively less (by approx. 10%) than for CPT_1 in parallel experiments with the same mitochondrial preparations.

Malonyl-CoA inhibits overt COT activity in rat liver and heart mitochondria. Liver COT is more sensitive to malonyl-CoA than is liver CPT_1, and the sensitivity of COT is not affected by starvation (Saggerson & Carpenter, 1981*a*). Heart overt COT is inhibited by malonyl-CoA concentrations similar to those that inhibit heart CPT_1, but only approx. 50% of this COT activity is inhibitable (Saggerson & Carpenter, 1981*b*). The pattern of inhibition of overt COT by bromoacetyl-CoA

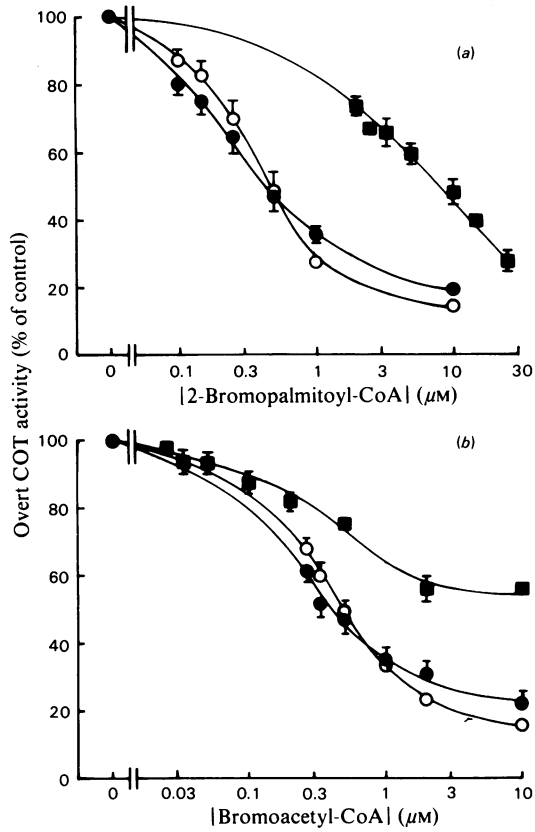

Fig. 4. *Effects of* DL-*2-bromopalmitoyl-CoA and bromoacetyl-CoA on overt COT activities*

Overt COT assays were performed as described in the Materials and methods section. L-Carnitine and octanoyl-CoA were present throughout at 400μM and 40μM respectively. COT activities with these substrates alone were 5.95 ± 0.75, 12.58 ± 1.65 and 4.07 ± 1.02nmol/min per mg of protein for mitochondria from fed-rat liver, 24h-starved-rat liver and heart respectively. All values are expressed as mean percentages of these activities in the absence of inhibitory acyl-CoAs. The bars indicate S.E.M. Where not shown, these lie within the symbol. The values in brackets indicate the numbers of separate experiments. (*a*) ●, liver (fed) [4]; ○, liver (24h starved) [3]; ■, heart (fed) [5]. (*b*) Symbols as for (*a*) [3 in each case].

Table 2. *IC_{50} and $I_{max.}$ values for inhibition of COT_1 by* DL-*2-bromopalmitoyl-CoA and bromoacetyl-CoA*
The values for each experiment were calculated from the data of Figs. 4(*a*) and 4(*b*) as described in the Materials and methods section, and are means ± S.E.M. for the numbers of experiments shown in brackets. *indicates significantly different IC_{50} value ($P<0.01$) from that found in fed liver.

	Liver (fed)		Liver (24h starved)		Heart (fed)	
Inhibitors	IC_{50}(μM)	$I_{max.}$ (%)	IC_{50}(μM)	$I_{max.}$ (%)	IC_{50}(μM)	$I_{max.}$ (%)
DL-2-Bromopalmitoyl-CoA	0.31 ± 0.05	84 ± 3 [4]	0.36 ± 0.05	89 ± 2 [3]	4.2 ± 0.6*	77 ± 3 [5]
Bromoacetyl-CoA	0.26 ± 0.04	80 ± 5 [3]	0.44 ± 0.10	92 ± 3 [3]	0.25 ± 0.05	44 ± 5 [3]

shown in Fig. 4(*b*) and Table 2 is similar to these previously reported effects of malonyl-CoA. The IC_{50} with bromoacetyl-CoA was significantly lower for COT than for CPT_1 in liver from both fed ($P<0.05$) and starved rats ($P<0.02$), but not significantly different in heart ($0.05<P<0.1$). The maximum inhibitable overt COT activity in heart was only approximately half of the total. Since bromoacetyl-CoA is a potent active-site-directed inhibitor of carnitine acetyltransferase (Chase & Tubbs, 1969), this non-inhibitable COT activity cannot be attributed to carnitine acetyltransferase. Likewise, since approx 90% of the heart CPT_1 is inhibitable by bromoacetyl-CoA (Fig. 1*b*) (presumably through an interaction with the malonyl-CoA site), it seems unlikely that this uninhibitable COT could be CPT. It is therefore concluded that the heart preparation contains a third overt acyltransferase activity distinct from CPT_1 and carnitine acetyltransferase. This conclusion is at variance with the findings of Clarke & Bieber (1981).

Displacement of [2-^{14}C]malonyl-CoA from mitochondrial binding sites by 2-bromopalmitoyl-CoA and bromoacetyl-CoA

Intuitively, it would seem most likely that 2-bromopalmitoyl-CoA is an inhibitor of CPT_1 by virtue of an interaction with the acyl-CoA-binding site of the enzyme. If this is so, then the major differences observed in the mode of inhibition by malonyl-CoA compared with 2-bromopalmitoyl-CoA should support the hypothesis that malonyl-CoA acts at a separate site. These major differences are as follows. First, 2-bromopalmitoyl-CoA potently inhibits the solubilized liver enzyme (West *et al.*, 1971; Hoppel & Tomec, 1972), whereas solubilization of the liver enzyme desensitises it to malonyl-CoA (McGarry *et al.*, 1978). Second, the inhibitory potency of 2-bromopalmitoyl-CoA is unaffected by Nbs_2 (Table 1), whereas this thiol-group reagent considerably desensitizes CPT_1 to malonyl-CoA (Saggerson & Carpenter, 1982*b*). Third, starvation does not decrease the sensitivity of liver CPT_1 to 2-bromopalmitoyl-CoA (Fig. 1*a*; Table 1), whereas sensitivity to malonyl-CoA (Saggerson & Carpenter, 1981*a*) and bromoacetyl-CoA (Fig. 1*b*; Table 1) is decreased. Fourth, when heart and liver CPT_1 activities are compared, the relative potency for inhibition by 2-bromopalmitoyl-CoA is the reverse of that for inhibition by malonyl-CoA (Saggerson & Carpenter, 1981*b*) or bromoacetyl-CoA (Table 1).

Palmitoyl-CoA displaces [^{14}C]malonyl-CoA from binding sites on skeletal muscle, heart and liver mitochondria (Mills *et al.*, 1983, 1984; Bird & Saggerson, 1984). By itself this observation cannot differentiate between the possibilities either that the displacement is from the acyl-CoA substrate-binding site or that it is from a separate malonyl-CoA-binding site, although the finding that raising pH decreases the K_D for palmitoyl-CoA to compete for malonyl-CoA binding without changing the K_m for palmitoyl-CoA (Mills *et al.*, 1984) supports the latter possibility. Fig. 5 demonstrates that 2-bromopalmitoyl-CoA displaced [^{14}C]malonyl-CoA from liver mitochondria. In a subsidiary experiment it was established that the tested concentration range of 2-bromopalmitoyl-CoA did not cause appreciable damage to the mitochondria. This was established by measuring overt and total (after sonication) glutamate dehydrogenase activity under the conditions of the binding assay. Over a range of [2-bromopalmitoyl-CoA] of 0–60 μM only 6% of the glutamate dehydrogenase was overt. This value was increased to 8.5% and to 12% with 80 μM and 100 μM-2-bromopalmitoyl-CoA respectively. The I_{50} for displacement by bromopalmitoyl-CoA (0.1 μM-malonyl-CoA present) was not greatly influenced by the absence or presence of carnitine (I_{50} values of 16 μM and 20 μM respectively) and was similar to the I_{50} value of 22 μM for the displacement of 0.1 μM-malonyl-CoA by palmitoyl-CoA from liver mitochondria (Bird & Saggerson, 1984). L-Carnitine alone caused some displacement of [^{14}C]malonyl-CoA from liver mitochondria, a phenomenon which is described more fully in the accompanying paper (Bird & Saggerson, 1985). The K_D for [^{14}C]malonyl-CoA binding to high-affinity sites on liver mitochondria from fed rats

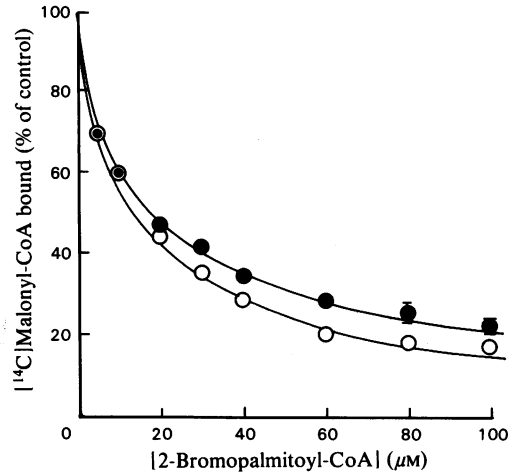

Fig. 5. *Effect of* DL-*2-bromopalmitoyl-CoA on* [*2-*^{14}*C*]*malonyl-CoA binding to fed-rat liver mitochondria*
Binding was measured as described in the Materials and methods section, with 0.1 μM-[2-^{14}C]malonyl-CoA and the indicated concentrations of 2-bromopalmitoyl-CoA. The values are means ± S.E.M. for three experiments: ○, without L-carnitine; ●, with L-carnitine (400 μM). Absolute values for binding in the absence of 2-bromopalmitoyl-CoA were: without L-carnitine, 9.2 ± 1.5 pmol/mg of protein; with L-carnitine, 6.7 ± 0.6 pmol/mg of protein.

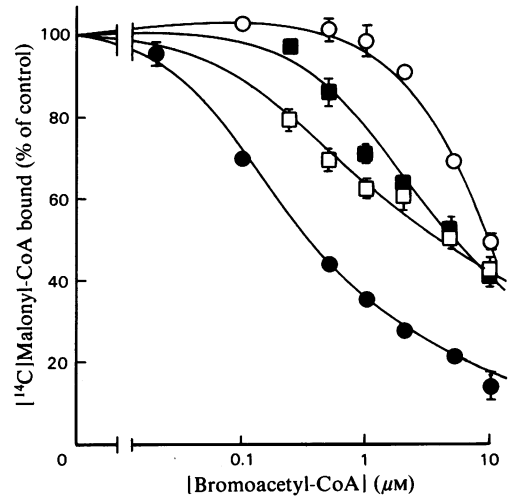

Fig. 6. *Effect of bromoacetyl-CoA on* [*2-*^{14}*C*]*malonyl-CoA binding to liver and heart mitochondria from fed rats*
Binding was measured as described in the Materials and methods section with 0.05 μM-[2-^{14}C]malonyl-CoA with heart mitochondria or 0.1 μM-[2-^{14}C]-malonyl-CoA with liver mitochondria and the indicated concentrations of bromoacetyl-CoA. All values are the means for three experiments. The bars represent S.E.M. Where these are not shown, they lie within the symbol. □, Liver, without L-carnitine; ■, liver with L-carnitine (400 μM); ○, heart, without L-carnitine; ●, heart, with L-carnitine (400 μM). Absolute values for binding in the absence of bromoacetyl-CoA were: liver, 9.7 ± 0.4 and 6.3 ± 0.3 pmol/mg of protein in the absence and presence of carnitine respectively: heart, 5.3 ± 0.3 and 5.7 ± 0.2 pmol/mg of protein in the absence and presence of carnitine respectively.

under conditions identical with those used here is 0.1 μM (Bird & Saggerson, 1984). By using the equation derived by Cheng & Prúsoff (1973), which states that:

$$K_D \text{ (competing ligand)} = \frac{I_{50} \text{ (competing ligand)}}{\dfrac{1+[[^{14}\text{C}]\text{malonyl-CoA}]}{K_D \text{ (malonyl-CoA)}}} \quad (1)$$

it can be calculated that the K_D for 2-bromopalmitoyl-CoA competing at the malonyl-CoA site is 8 or 10 μM in the absence or presence of carnitine respectively. If this site were the catalytic site, the K_D value should be less than the IC_{50} for inhibition of liver CPT_1 by 2-bromopalmitoyl-CoA. However, the IC_{50} value was approx. 25-fold smaller (Table 1), and it is concluded that this experiment provides further evidence that the high-affinity malonyl-CoA-binding site is distinct from the catalytic site or, more specifically, the acyl-CoA substrate-binding Site. The difference between the effects of bromopalmitoyl-CoA in inhibiting CPT_1 and in displacing bound malonyl-CoA was clearly demonstrated by incubation of liver mitochondria for 20 min under conditions identical with those used in the binding assays with 0, 5 μM and 20 μM inhibitor. The incubations were diluted 20-fold and CPT_1 assays performed. The resulting inhibitions were 72% and 90% for 5 μM- and 20 μM-bromopalmitoyl-CoA respectively, whereas the displacement of bound [^{14}C]malonyl-CoA (0.1 μM) was only approx. 30% and 50% respectively in parallel binding experiments.

If, as discussed in the previous section, malonyl-CoA and bromoacetyl-CoA interact at the same site, bromoacetyl-CoA should displace [^{14}C]-malonyl-CoA from high-affinity binding sites. This was observed (Fig. 6), but not entirely in the manner expected. Bromoacetyl-CoA displaced [^{14}C]malonyl-CoA from liver mitochondria in the presence and absence of carnitine. Carnitine had little effect on the percentage displacement curves in liver, although, by itself, carnitine caused some decrease in malonyl-CoA binding (Bird & Saggerson, 1985). In view of its greater inhibitory potency against heart compared with liver CPT_1 (Table 1),

it was therefore surprising to find that bromoacetyl-CoA by itself was rather ineffective at displacing 50nM-[^{14}C]malonyl-CoA from heart mitochondria (Fig. 6), where 50% displacement was only achieved with 10μM-bromoacetyl-CoA. The K_D for malonyl-CoA binding to heart mitochondria high-affinity sites under conditions identical with those used here is 11nM (Bird & Saggerson, 1984) which, when fitted to eqn. (1), would yield a K_D value for bromoacetyl-CoA at this site of 1.8μM. This is an order of magnitude higher than the IC_{50} for inhibition of heart CPT_1 by bromoacetyl-CoA (Table 1), and clearly is anomalous. However, inclusion in the binding assay of carnitine (which alone has no effect on malonyl-CoA binding in heart, unlike liver; Bird & Saggerson, 1985) caused a 30-fold potentiation of malonyl-CoA displacement by bromoacetyl-CoA, such that the I_{50} was now 0.34μM. By using $K_D = 15$nM for [^{14}C]malonyl-CoA binding in the presence of 400μM-carnitine (Fig. 7), the K_D for bromoacetyl-CoA at the heart malonyl-CoA site in the presence of carnitine was found to be 78nM, which is not inconsistent with the IC_{50} for inhibition of heart CPT_1 of 140nM (Table 1). Fig. 7 shows a Scatchard analysis of [^{14}C]malonyl-CoA binding to heart mitochondria over a concentration range which essentially only encompasses interaction with high-affinity sites (Bird & Saggerson, 1984). The K_D for this binding, which was performed in the presence of 400μM-carnitine, was not different from that observed in the absence of carnitine by Bird & Saggerson (1984). Fig. 7 also shows that bromoacetyl-CoA (in the presence of carnitine) appeared to interact with this binding site in a competitive manner with malonyl-CoA. It was concluded from this series of experiments that carnitine considerably facilitated competition by bromoacetyl-CoA at the high-affinity malonyl-CoA-binding site in heart mitochondria. It is also noteworthy that Fig. 6 demonstrates a further inter-tissue difference between liver and heart, in that carnitine does not appear to facilitate such an effect of bromoacetyl-CoA in liver mitochondria. In view of the finding that bromoacetyl-CoA causes an extremely persistent active-site-directed inhibition of carnitine acetyltransferase when carnitine is present (Chase & Tubbs, 1969), the experiment summarized in Table 3 was performed. This demonstrated clearly that, when heart mitochondria were washed after exposure to 5μM-bromoacetyl-CoA (without or with carnitine), no change in the CPT_1 activity, [^{14}C]malonyl-CoA binding or the response to further treatment with bromoacetyl-CoA persisted. Furthermore, if heart mitochondria were incubated on ice for 10min with 5μM-bromoacetyl-CoA (without or with

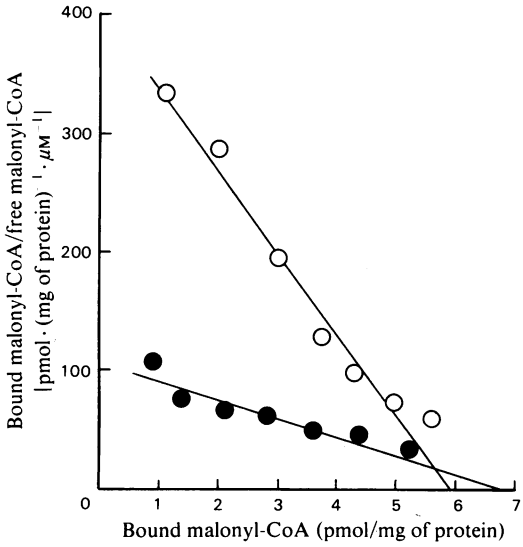

Fig. 7. *Scatchard analysis showing displacement of [^{14}C]malonyl-CoA from high-affinity binding sites in heart mitochondria by bromoacetyl-CoA*

Binding was measured as described in the Materials and methods section with 5nM-, 10nM-, 20nM-, 35nM-, 50nM-, 75nM- and 100nM-[2-^{14}C]malonyl-CoA; 400μM-L-carnitine was present throughout. The values are means of two experiments: ○, without bromoacetyl-CoA; ●, with 150nM-bromoacetyl-CoA (150nM-bromoacetyl-CoA increased the K_D for malonyl-CoA binding from 15 to 65nM).

400μM-carnitine) and then simply diluted 20-fold and immediately assayed, the observed inhibition of CPT_1 was the same as in CPT_1 assays performed directly with 0.25μM-bromoacetyl-CoA (results not shown). It is therefore suggested that carnitine must act rapidly and reversibly to modify the environment of the malonyl-CoA-binding site so that reversible competition by bromoacetyl-CoA is facilitated. In this respect, it is particularly noteworthy that Mills *et al.* (1984) have suggested that the carnitine- and the malonyl-CoA-binding sites on CPT_1 are closely associated. This suggestion arose because of the reciprocal relationship in heart, skeletal muscle and liver between the inhibitory potency of malonyl-CoA and the K_m for carnitine (Mills *et al.*, 1983, 1984).

Finally, the displacement of [^{14}C]malonyl-CoA by 2-bromopalmitoyl-CoA was also investigated with heart mitochondria (Fig. 8). Again, although carnitine alone had no effect on malonyl-CoA binding (also see Bird & Saggerson, 1985), 400μM-carnitine potentiated the displacement by 2-bromopalmitoyl-CoA by approx. 3-fold. This may be contrasted with Fig. 5, in which no potentiating

Table 3. *Reversibility of effects of bromoacetyl-CoA in heart mitochondria*

Heart mitochondria from fed animals (2.9mg of protein/ml) were preincubated for 10min at 0–4°C in 1.5ml of 25mM-Tris/HCl buffer (pH7.4) containing 150mM-sucrose, 60mM-KCl, fatty acid-poor albumin (1.3mg/ml), 1mM-EGTA and: (*a*) no further additions; (*b*) 5μM-bromoacetyl-CoA; or (*c*) 5μM-bromoacetyl-CoA + 400μM-L-carnitine. The mitochondria were then sedimented by centrifugation for 2min at 6500 $g_{av.}$ in an Eppendorf 5412 centrifuge (at 4°C) and the supernatant was discarded. The pellet was resuspended in 1.5ml of ice-cold 0.3M-sucrose medium containing 10mM-Tris/HCl buffer (pH7.4) and 1mM-EGTA and re-centrifuged. This washing procedure was repeated, and the mitochondria were finally resuspended in 1.5ml of the 0.3M-sucrose medium. Portions (50μl and 300μl respectively) were taken for CPT_1 assay and measurement of [^{14}C]malonyl-CoA binding as described in the Materials and methods section.

Preincubation conditions	CPT_1 activity at 40μM-palmitoyl-CoA (nmol/min per mg of protein)	[2-^{14}C]Malonyl-CoA binding with 50nM-malonyl-CoA (pmol/mg of protein)	IC_{50} for bromoacetyl-CoA (μM)
a	2.9	4.6	0.35
b	3.1	4.2	0.25
c	2.8	4.1	0.25

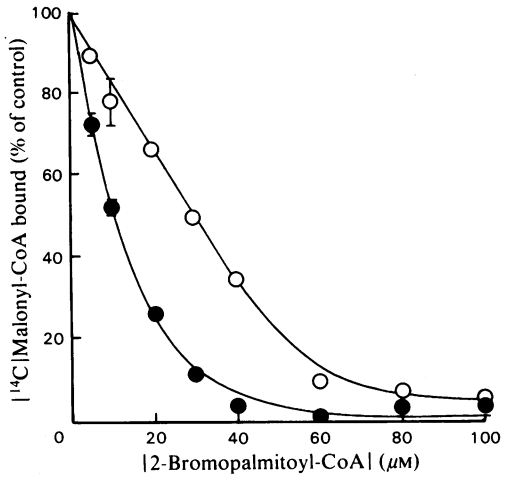

Fig. 8. *Effect of* DL-*2-bromopalmitoyl-CoA on [2-^{14}C]-malonyl-CoA binding to heart mitochondria form fed animals*

Binding was measured as described in the Materials and methods section with 0.05μM-[2-^{14}C]malonyl-CoA and the indicated concentrations of 2-bromopalmitoyl-CoA. The values are means of two experiments. The bars indicate the range of values. Where these are not shown, they lie within the symbol: ○, without L-carnitine; ●, with L-carnitine (400μM). Absolute values (±the range) for binding in the absence of 2-bromopalmitoyl-CoA were: without L-carnitine, 7.4±0.2pmol/mg of protein; with L-carnitine, 7.3±0.2pmol/mg of protein.

effect of carnitine was observed in liver mitochondria.

Conclusions

The principal conclusions are as follows. First, the studies with 2-bromopalmitoyl-CoA using liver mitochondria provide further evidence that the malonyl-CoA site is probably distinct from the acyl-CoA substrate-binding site of CPT_1. Second, it is demonstrated that bromoacetyl-CoA, like other short-chain CoA thioesters, is likely to interact at the malonyl-CoA site of CPT_1. It is noteworthy that bromoacetyl-CoA is the first ligand reported to bind to the malonyl-CoA inhibitory site as tightly as or more tightly than malonyl-CoA itself. Third, evidence is provided that there is likely to be interaction between the site for carnitine binding and the malonyl-CoA site. These data complement those in the accompanying paper (Bird & Saggerson, 1985). Fourth, further inter-tissue differences are revealed regarding both the difference in the inhibitory potency of 2-bromopalmitoyl-CoA and the effect of carnitine to facilitate displacement of malonyl-CoA by both bromoacetyl-CoA and bromopalmitoyl-CoA.

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